Pelvic inflammatory disease in patients infected with *Chlamydia trachomatis:* in vitro cell mediated immune response to chlamydial antigens

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SUMMARY   Blood samples were obtained from 11 women with laparoscopically confirmed acute salpingitis who yielded positive cultures of *Chlamydia trachomatis* from the cervix. Four patients also had perihepatitis. Lymphocyte transformation assays, using *C trachomatis* serovars I and L2 as antigens, showed that the patients' lymphocytes responded to antigenic stimulation more strongly than the lymphocytes of age matched controls. Responses to the I and L2 antigens correlated strongly, but greater responses were obtained to the L2 antigen. No correlation was found between the response in the lymphocyte transformation assay and the degree of the inflammatory changes of the fallopian tubes, the presence of perihepatitis, or the titres of humoral antibodies to *C trachomatis* as measured by microimmunofluorescence. Sequential transformation assays, however, showed that patients with perihepatitis tended to have a more sustained response.

Introduction

Chlamydia trachomatis is a common cause of genital tract infections in women, such as cervicitis, endometritis, and salpingitis.[1][2] Genital chlamydial infections generally produce circulating as well as local antibodies, which are detectable by the microimmunofluorescence method of Wang and Grayston.[3] Chlamydial infections of the cervix have also been shown to induce a cell mediated immune response to chlamydial antigens.[4][5]

The relative importance of humoral and cell mediated immunity in the pathophysiology of chlamydial infections is not well known. Cell mediated immunity might be expected to be important to clear an intracellular micro-organism such as *C trachomatis*. On the other hand, extrapolating from evidence in ocular chlamydial infections, cell mediated immune reactions might also contribute to tissue damage in chlamydial infections of the genital tract.[5]

In the study reported here 11 women with laparoscopically confirmed salpingitis who yielded cultures of *C trachomatis* from the cervix were tested for immune response to chlamydial antigens using a lymphocyte stimulation test in vitro. The results were compared with those of age matched controls and analysed in relation to the humoral antibody response to *C trachomatis,* the severity of the tubal disease, and the occurrence of perihepatitis.

Patients, materials, and methods

PATIENTS AND CONTROLS

We studied 11 patients who were admitted to the department of obstetrics and gynaecology of Lund University Hospital during 1981-2. They were derived from a series of women who had laparoscopically confirmed acute salpingitis. The laparoscopic technique and the criteria for grading the inflammatory changes of the fallopian tubes have been described elsewhere.[6] We included in the study only patients who were culture positive for *C trachomatis* but not for *Neisseria gonorrhoeae.* Nine age matched controls were selected from women members of the hospital staff who to their knowledge had never had a chlamydial infection or acute salpingitis.

SAMPLING AND CULTURE PROCEDURES

We collected specimens for the isolation of *C trachomatis* and *N gonorrhoeae* from the cervix, the

urethra, and (in four patients) from the fimbriated end of the fallopian tubes as described previously.[7] Rectal specimens were also taken for culture for gonococci. In patients with laparoscopic evidence of perihepatitis, we also took samples from the surface of the liver with a specially designed, long, cotton tipped aluminium swab, and cultured them for *C trachomatis* and *N gonorrhoeae*.[8] We used previously reported methods to isolate and identify *C trachomatis*[9] and *N gonorrhoeae*.[10]

BLOOD SAMPLING
We collected blood samples for lymphocyte transformation assays and to detect chlamydial antibodies 1-4 weeks (from eight patients) or 5-7 weeks (from three patients) after the onset of lower abdominal pain. In nine patients and eight controls a second sample was collected 4-6 weeks (one week in one test) later for a second lymphocyte transformation assay. To measure serum chlamydial antibodies, we took two or more samples from 10 patients during the acute phase (within three weeks of the onset of symptoms).

DETECTING HUMORAL ANTIBODIES
We used a modified microimmunofluorescence test to detect serum IgG and IgM antibodies to *C trachomatis*.[11] Each serum sample was tested for antibodies against three pools of antigen: A-C, D-K, and L1-3. Paired samples from patients when they were acutely ill and convalescent were analysed simultaneously.

LYMPHOCYTE TRANSFORMATION ASSAY
Chlamydial antigens
Immunotypes I and L2 of *C trachomatis* (kindly supplied by Dr S-P Wang, University of Washington, Seattle, USA) were cultivated in the yolk sac of embryonated hens' eggs until 50% of the embryos had died. The yolk sacs were then harvested from the living eggs, shaken with glass beads, washed, and frozen in aliquots containing about 5×10^6 inclusion forming units (ifu)/ml. Working dilutions of 1/10, 1/40, and 1/160, which corresponded to 50×10^3, 12×10^3 and 3×10^3 ifu per 10^5 lymphocytes were used for the lymphocyte transformation assay (see below). Uninfected yolk sac, treated as above, was used as a control.

Lymphocytes
About 30 ml heparinised blood were mixed with carbonyl iron particles in methyl cellulose, and the mixture was rotated at 37°C for 30 minutes. An equal volume of saline was added, and the diluted blood was centrifuged on Isopaque-Ficoll (Lymphoprep, Nyegaard Co, Norway) at $400 \times g$ for 30 minutes.

Cells at the interphase were collected, washed three times in tissue culture medium, and suspended in RPMI 1640 medium (Flow Laboratories, Solna, Sweden) buffered with N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) supplemented with glutamine and 10% human group AB serum (negative for *C trachomatis* on microimmunofluorescence).

Cultivation procedure
Quadruplicate samples of 10^5 lymphocytes in 100 μl of tissue culture medium with an appropriate dilution of antigen (final dilution 1/10, 1/40, or 1/160) were cultured for 120 hours in microplates (No 76-213-05, Flow Laboratories) at 37°C in 7% carbon dioxide. The incorporation of tritiated thymidine into lymphocytes was measured during the final four hours after adding 37 kBq (1 μCi) ^{3}H-Tdr (TRK 120, Amersham Sweden AB, Solna, Sweden) per well. The cells were harvested on glass fibre filters by means of a microplate cell harvester (Titertek Cell Harvester, Flow Laboratories), and their activity was calculated by liquid scintillation counting.

Presentation of data
All calculations were performed on counts per minute (cpm) transformed by $\log_{10}$. Non-parametric tests were used for statistical analysis.

Results

Table I shows the laparoscopic and serological findings in the 11 patients, four of whom were classified as having mild inflammatory changes of the fallopian tubes, whereas seven had moderately severe changes. Four had perihepatitis in addition to salpingitis.

The serological tests showed a fourfold or more change in titres of IgG antibody to *C trachomatis* in four patients. Two of the remaining women (Nos 4 and 6), both with perihepatitis, had stationary high titres during the observation period. The remaining five patients had lower stationary titres (1/256 to 1/1024). IgM antibodies (titres 1/32 to 1/256) were detected in samples taken during the acute phase from three of the patients.

C trachomatis in the cervix was one of the criteria for inclusion in the study. Chlamydiae were cultured from the surface of the liver of one of the patients with perihepatitis and from the fallopian tubes of one of four patients.

Tables II and III show the results of the lymphocyte transformation assays with *C trachomatis* I and L2 antigens. Lymphocytes from the patients showed a significantly greater response than those from the controls at all antigen dilutions tested.

TABLE I *Clinical data of 11 patients with salpingitis and results of serological tests for Chlamydia trachomatis*

Case No	Age (years)	Grade of salpingitis	Duration of symptoms (weeks)*	Perihepatitis	Titre of IgG antibody to C trachomatis at:	
					Acute phase	Convalescence
1	15	Mild	1	+	1/512	1/512
2	19	Mild	2		1/16	1/1024
3	20	Mild	2		1/256	1/256
4	29	Mild	5	+	1/4096	1/2048
5	18	Moderately severe	2		1/512	1/2048
6	19	Moderately severe	4	+	1/8000	1/16000
7	22	Moderately severe	7		1/1024	NT
8	25	Moderately severe	6	+	1/256	1/4096
9	25	Moderately severe	3		1/128	1/256
10	36	Moderately severe	2		1/8000	1/512
11	40	Moderately severe	4		1/512	1/1024

*At first lymphocyte transformation test.
NT = not tested.

Correlation analysis of cpm transformed by $\log_{10}$ showed that the optimum responses to the two antigens were closely associated (Spearman's rank correlation coefficient, r = 0·98; Wilcoxon signed rank test, z = 2·93, p<0·01). However, comparison between the responses obtained in individual patients with the two immunotypes indicated certain differences. Thus antigen L2 gave a greater response than antigen I in all 11 patients at a dilution of 1/10, and in nine patients at a dilution of 1/40. At a dilution of 1/160 no such difference was seen.

Lymphocyte transformation assays were repeated 4-6 weeks after the first assay in eight patients and after one week in one patient. Four of these patients

TABLE II *Lymphocyte stimulation in vitro by Chlamydia trachomatis immunotype I antigen in patients and controls (mean (SD) $\log_{10}$ counts per minute)*

	Patients (n = 11)	Controls (n = 9)	p value*
Antigen dilution:			
1/10	3·86 (0·40)	2·93 (0·57)	<0·01
1/40	4·23 (0·34)	3·36 (0·64)	<0·01
1/160	4·14 (0·47)	3·21 (0·61)	<0·01
Culture medium with uninfected yolk sac	2·87 (0·51)	2·76 (0·46)	NS

*Mann-Whitney U test.

also had perihepatitis, whereas the remaining five had salpingitis only. Eight control subjects, who were age matched with the patients, were also tested again on this second occasion. The figure shows the results of the tests on both occasions. In four out of five patients with uncomplicated salpingitis the responses decreased between the first and second tests, whereas in three patients with perihepatitis the responses increased. The fourth patient with perihepatitis (No 4) showed a high response (about 45 000 cpm) in the first test, and the response remained high for at least six weeks. The first sample had not been taken from

TABLE III *Lymphocyte stimulation in vitro by Chlamydia trachomatis immunotype L2 antigen in patients and controls (mean (SD) $\log_{10}$ counts per minute)*

	Patients (n = 11)	Controls (n = 9)	p value*
Antigen dilution:			
1/10	4·21 (0·37)	3·14 (0·50)	<0·001
1/40	4·36 (0·37)	3·34 (0·51)	<0·001
1/160	4·10 (0·53)	3·14 (0·52)	<0·01
Culture medium with uninfected yolk sac	2·87 (0·51)	2·76 (0·46)	NS

*Mann-Whitney U test.

this patient until five weeks after the onset of abdominal pain.

Discussion

The investigation reported here showed that patients with laparoscopically confirmed salpingitis who were infected with *C trachomatis* developed an immune
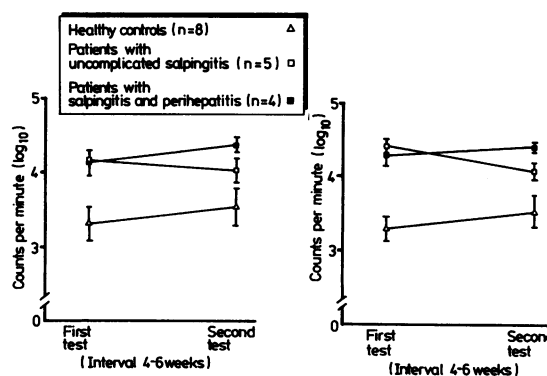

FIG *Tests for lymphocyte transformation in vitro repeated at intervals of 4-6 weeks using Chlamydia trachomatis I antigen (left) and L2 antigen (right). (Y axis shows mean (SE) incorporation of ^{3}H-thymidine).*

response that was demonstrable by lymphocyte transformation in vitro with chlamydial antigens I and L2.

Strains of *C trachomatis* that are responsible for uncomplicated genital infections (Saikku, personal communication) and pelvic inflammatory disease (Mårdh P-A, unpublished observation) in Scandinavian patients may belong to any of the serotypes D-K. Our patients showed very similar serum antibody titres to the D-K and L1-L3 antigen pools on microimmunofluorescence, whereas titres to immunotypes A-C were generally one or two dilution steps lower (data not shown). The microimmunofluorescence test has shown extensive cross reactions between the L2 antigen and other serovars,[12 13] and in lymphocyte transformation assays responses to the L2 and E antigens have been found to correlate in patients with cervicitis.[4]

In the study reported here we found a correlation between lymphocyte responses to the I and L2 antigens, with a higher response to the L2 antigen than to the I antigen. Optimum lymphocyte transformation responses to both I and L2 antigens were obtained at the intermediate dilution of antigen (1/40), which indicated that differences in response were not caused by different numbers of ifu in the two antigen preparations. Better adsorption to cell surfaces by both living and dead L particles than by other serovars[14] may explain the higher response to the L2 antigen.

Three of the control subjects showed a definite lymphocyte transformation response (ten times or more above background) to chlamydial antigens I and L2. This was not an unexpected finding, as infections with this agent are widespread in the population of the study area.[15] Hanna *et al*[16] and Qvigstad *et al*[17] also found positive lymphocyte transformation responses to chlamydial antigens in some healthy volunteers, which they explained on the basis of previous asymptomatic infections with chlamydiae.

Positive lymphocyte transformation assays correlate with the presence of chlamydial antibodies in patients consulting sexually transmitted disease (STD) clinics and in asymptomatic volunteers.[4 16] In some patients antibody titres and lymphocyte transformation responses seemed to correlate quantitatively.[18] In our patients with acute salpingitis, the titres of humoral antibodies did not correlate with the level of the response in the lymphocyte transformation assay at any given time. Whether this means that the lymphocyte transformation assay measures a variable that is independent of the humoral antibody response is not clear. Brunham *et al*[5] and Qvigstad *et al*[17] provided evidence that T cells rather than B cells respond by transformation in vitro to chlamydial antigen. There

is still no evidence, however, that the lymphocyte transformation assay measures the degree of an immune response that is important for the outcome of the disease.

So far the relative importance of humoral versus cell mediated responses in the pathogenesis and course of chlamydial infections has not been elucidated.[19] Animal models have provided evidence both for and against cell mediated immunity playing a protective part in such infections.[20-23]

In our patients, the lymphocyte transformation response did not correlate with the grading of the inflammatory changes of the fallopian tubes seen at laparoscopy. Qvigstad *et al* also reported that in vitro responses of T cells to chlamydial antigens were similar in patients with salpingitis and those with cervicitis only.[24]

Immune mechanisms may participate in the development of perihepatitis associated with chlamydiae in women with pelvic inflammatory disease.[25] As reported earlier, patients can yield positive cultures for *C trachomatis* from the liver surface.[8] Thus a direct microbial invasion of the liver surface could well explain the pathogenesis of this syndrome. In the study reported here we found that patients with perihepatitis tended to have a more longstanding response in the lymphocyte transformation assay. This may be explained by more extensive immunisation in patients with perihepatitis than in those whose genital tract only is affected. We do not yet know whether (and, if so, to what extent) immunological mechanisms contribute to the tissue lesions.

More precise evaluation of the possible role of cell mediated immune reactions in the pathogenesis of salpingitis induced by *C trachomatis* requires studies in a suitable animal model. Such a model has been established in the grivet monkey.[26]

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